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Zoo Animals as Reservoirs of Gram-Negative Bacteria Harboring Integrons and Antimicrobial Resistance Genes[▽]

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A total of 232 isolates of gram-negative bacteria were recovered from mammals, reptiles, and birds housed at Asa Zoological Park, Hiroshima prefecture, Japan. Forty-nine isolates (21.1%) showed multidrug resistance phenotypes and harbored at least one antimicrobial resistance gene. PCR and DNA sequencing identified class 1 and class 2 integrons and many β-lactamase-encoding genes, in addition to a novel AmpC β-lactamase gene, bla_{CMY-26}. Furthermore, the plasmid-mediated quinolone resistance genes *qnr* and *aac(6′)-Ib-cr* were also identified.

Problems associated with the development and spread of antibiotic resistance in clinical practice have been increasing since the early 1960s and are currently viewed as a major threat to the public health on a global level (15). Animals, particularly wild animals, are believed to be the source of >70% of all emerging infections (13). A recent report identified more than 25 human infectious disease outbreaks over a 10-year period (1990 to 2000) as being associated with visits to animal exhibits (2). Of particular concern is the potential transmission of multidrug-resistant zoonotic pathogens from zoo animals to humans. As little is known about antimicrobial-resistant bacteria in zoo animals, this study was conducted to monitor the incidence and prevalence of antimicrobial resistance genes in gram-negative bacteria isolated from zoo animals in Japan.

A total of 103 swabs (68 fecal, 33 water, and 2 nasal swabs) were randomly taken from different mammals, reptiles, birds, and water sources between June and September 2006 at Asa Zoological Park, Hiroshima prefecture, Japan. A total of 232 gram-negative bacteria were isolated, and the biochemical identification showed that the most prevalent species was *Escherichia coli* (122 isolates; 52.6%), followed by *Klebsiella pneumoniae* (17 isolates; 7.3%), *Proteus mirabilis* (16 isolates; 6.9%), *Enterobacter aerogenes* (14 isolates; 6.0%), *Klebsiella oxytoca* (13 isolates; 5.6%), *Pseudomonas aeruginosa* (12 isolates; 5.2%), *Enterobacter cloacae* (11 isolates; 4.7%), *Proteus vulgaris* (5 isolates; 2.2%), *Citrobacter koseri* (5 isolates; 2.2%), *Citrobacter freundii* (4 isolates; 2.2%), *Morganella morganii* (4 isolates; 1.7%), *Salmonella* spp. (3 isolates; 1.3%), *Serratia marcescens* (2 isolates; 0.9%), and a single isolate (0.43%) of *Acinetobacter baumannii*, *Aeromonas* spp., *Pseudomonas fluorescens*, and *Edwardsiella tarda*.

The antimicrobial sensitivity phenotypes of recovered bacteria were determined by using a disk diffusion assay according to the standards and interpretive criteria described by CLSI (5). The results showed that 49 isolates (21.1%) showed resistance phenotypes to two or more antimicrobial agents. The most commonly reported resistance phenotypes were against ampicillin, cephalothin, streptomycin, trimethoprim-sulfamethoxazole, kanamycin, tetracycline, nalidixic acid, and ciprofloxacin. Similar resistance phenotypes have been recorded previously for strains of *E. coli* isolated from wild animals in Portugal, from free-living Canada geese in Georgia and North Carolina, and from black-headed gulls in the Czech Republic (6–8). Interestingly, many isolates showed resistance phenotypes to extended-spectrum β-lactam antibiotics, such as cefotaxime, ceftazidime, cefpodoxime, ceftriaxone and aztreonam, which are widely used for the treatment of serious infections in hospitals (3).

Integrons play a major role in the spread of antibiotic resistance genes in gram-negative bacteria (28). In this study, primers 5′-CS and 3′-CS, which amplify the region between the 5′ conserved segment and 3′ conserved segment of class 1 integrons, were used as previously described (Table 1) (14). PCR screening detected class 1 integrons in 16 bacterial isolates (6.9%); 11 *E. coli* isolates, 2 *P. vulgaris* isolates, and 1 isolate of *E. cloacae*, *M. morganii*, and *P. mirabilis* (Table 2). DNA sequencing results for the inserted gene cassettes identified seven profiles of class 1 integrons (Table 2). The identified antimicrobial resistance genes were *dfrA1*, *dfrA5*, *dfrA12*, *dfrA15*, and *dfrA17*, dihydrofolate reductase types which confer resistance to trimethoprim, and *aadA1*, *aadA2* and *aadA5*, aminoglycoside adenyltransferase types which confer resistance to streptomycin and spectinomycin. The resistance phenotypes were expressed for most of these genes (Table 2). It was of interest that class 1 integrons harboring *aadA1* have been previously identified for *E. coli* isolated from free-living Canada geese in Georgia and North Carolina and black-headed gulls in the Czech Republic (6, 8), while another type of class 1 integron harboring *aadA7* has been previously identified in an *E. coli*

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TABLE 1. Primers used in this study

Target category and primer	Sequence (5′ to 3′)	Target	Reference or GenBank accession no.
Integrons			
5′-CS	GGCATCCAAGCAGCAAG	Class 1 integron	14
3′-CS	AAGCAGACTTGACCTGA		
hep74	CGGGATCCCGGACGGCATGCACGATTTGTA	Class 2 integron	31
hep51	GATGCCATCGCAAGTACGAG		
IntI2-F2	GATCCTGCCATCATTGAGTA	Within class 2 integron	1
IntI2-R2	AGGGGAAGCCGAAGTTTCC		
β-Lactamases			
TEM-F	ATAAAATTCTTGAAGACGAAA	bla_{TEM}	1
TEM-R	GACAGTTACCAATGCTTAATC		
SHV-F	TTATCTCCCTGTTAGCCACC	bla_{SHV}	1
SHV-R	GATTTGCTGATTTCGCTCGG		
SHV-F-2	CGGCCTTCACTCAAGGATGTA	Whole bla_{SHV}	DQ247972
SHV-R-2	GTGCTGCGGGCCGGATAAC		
OXA-F	TCAACTTTCAAGATCGCA	bla_{OXA}	1
OXA-R	GTGTGTTTAGAATGGTGA		
CTX-M-F	CGCTTTGCGATGTGCAG	bla_{CTX-M}	1
CTX-M-R	ACCGCGATATCGTTGGT		
CTX-M-F2	CCAGAATAAGGAATCCCATG	Whole bla_{CTX-M}	NC_004464
CTX-M-R2	GCCGTCTAAGGCGATAAAC		
CMY-F	GACAGCCTCTTTCTCCACA	bla_{CMY}	33
CMY-R	TGGAACGAAGGCTACGTA		
CMY-F2	ACGGAACTGATTTCATGATG	Whole bla_{CMY}	AY899928
CMY-R2	GAAAGGAGGCCCAATATCCT		
Oxy-F	GGTTTTGGTAACTGTGACGGG	bla_{OXY}	10
Oxy-R	CAGAGTGCAGAGTGTTGCAG		
Oxy-F2	GGCAATCCAGCCGGGGCCAA	Whole bla_{OXY}	Z30177
Oxy-R2	CGGGCCTGTTCCCGGGTTAA		
Plasmid-mediated quinolone resistance genes			
qnrA-F	ATTTCTCACGCCAGGATTTG	*qnrA*	27
qnrA-R	GATCGGCAAAGGTTAGGTCA		
qnrB-F	GATCGTGAAAGCCAGAAAGG	*qnrB*	27
qnrB-R	ACGATGCCTGGTAGTTGTCC		
qnrS-F	ACGACATTCGTCAACTGCAA	*qnrS*	27
qnrS-R	TAAATTGGCACCCTGTAGGC		
aac(6′)-Ib-F	TTGCGATGCTCTATGAGTGGCTA	*aac(6′)-Ib*	22
aac(6′)-Ib-R	CTCGAATGCCTGGCGTGTTT		

strain isolated from the Washington Zoo, Seattle, WA (17). On the other hand, for the detection of class 2 integrons, PCR was performed with the primer pair hep74 and hep51, specific to the conserved regions of class 2 integrons, as described previously (Table 1) (31). Class 2 integrons were detected in four isolates (1.7%), including three *E. coli* isolates and one *P. mirabilis* isolate (Table 2). DNA sequencing results for the inserted gene cassettes within class 2 integrons identified the three classic resistance genes *dfrA1*, *sat2*, and *aadA1*, which are usually associated with transposon Tn*7* (11). To the best of our knowledge, this is the first report for class 2 integrons from zoo animals.

Resistance to β-lactam antibiotics in gram-negative bacteria is mediated primarily by β-lactamases (3, 23). The bacterial isolates were tested for TEM, SHV, CTX-M, OXA, and CMY β-lactamase-encoding genes by PCR using universal primers for the TEM, SHV, OXA, CTX-M and CMY families, as described previously (Table 1) (1, 33). Detection of the OXY β-lactamase-encoding gene in *K. oxytoca* was carried out as described previously (10). PCR and DNA sequencing screenings detected bla_{TEM-1}, a narrow-spectrum β-lactamase gene which confers resistance against penicillins and narrow-spectrum cephalosporins, in 19 isolates (8.2%), which included 16 isolates of *E. coli* and 1 isolate of *A. baumannii*, *P. mirabilis*, and *P. vulgaris* (Table 2). All these isolates showed an ampicillin and cephalothin resistance phenotype (Table 2). TEM β-lactamase has been previously detected in *E. coli* isolated from wild animals in Portugal (7) and from free-living Canada geese in Georgia and North Carolina and black-headed gulls in the Czech Republic (6, 8). bla_{OXY-2}, another narrow-spectrum β-lactamase, was identified in three strains of *K. oxytoca*. Interestingly, all three isolates are from reptiles (Japanese four-striped rat snake, Colombian rainbow boa, and giant salamander) (Table 2). bla_{OXY-2} is a *K. oxytoca*-linked β-lactamase that confers resistance to narrow-spectrum cephalosporins and, to a lesser extent, to broad-spectrum cephalosporins, such as cefoperazone, and to monobactams, such as aztreonam (9). To the best of our knowledge, this is the first report of the bla_{OXY} gene from zoo animals.

Recently, there has been a dramatic increase in the incidence and prevalence of extended-spectrum β-lactamases (ESBLs) (3, 23). In this study, bla_{SHV-36}, an ESBL-encoding

TABLE 2. Resistance phenotype and prevalence of integrons and resistance genes in gram-negative bacteria

Isolate	Animal(s) (source)	Bacteria	Resistance phenotype[a]	Integron/resistance gene(s)
AZ1-1	Wild birds (feces)	*A. baumannii*	AMP, CEF	$bla_{\text{TEM-1}}$
AZ2-3	Wild birds (feces)	*E. coli*	TET, STR, NAL, SXT	Class 2 (*dfrA1-sat2-aadA1*)
AZ5-3	Wild birds (water)	*E. coli*	AMP, CEF, SXT, CHL	Class 1 (*dfrA17-aadA5*), $bla_{\text{TEM-1}}$
AZ10-1	Scarlet macaw (feces)	*E. coli*	AMP, CEF, TET, STR, NAL, CIP, SXT, GEN, NOR,	Class 1 (*dfrA1-aadA1*), $bla_{\text{TEM-1}}$
AZ12-2	Turtle (water)	*P. fluorescens*	NAL, SXT	*qnrB*
AZ15-3	White pelican (water)	*E. coli*	TET, STR, SXT	Class 2 (*dfrA1-sat2-aadA1*)
AZ19-1	Rhesus monkey (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
AZ20-1	Horned owl (feces)	*E. coli*	TET, KAN, STR, NAL, SXT	Class 2 (*dfrA1-sat2-aadA1*)
AZ23-2	Falcon (feces)	*E. coli*	STR, TET, NAL, SXT	Class 1(*dfrA15-aadA1*), *qnrB*
AZ23-3	Falcon (feces)	*P. vulgaris*	TET, STR, NAL, SXT	Class 1 (*dfrA1-orf*)
AZ23-4	Falcon (feces)	*E. coli*	STR, SXT	Class 1 (*dfrA15-aadA1*)
AZ26-2	Jaybird (feces)	*K. oxytoca*	FOX, CTT, AMP, CEF, CRO, AMC, SXT, KAN	$bla_{\text{CMY-26}}$
AZ26-4	Jaybird (feces)	*E. coli*	TET, STR, NAL, CIP, AMP, SXT, CHL, NOR	Class 1 (*aadA2*)
AZ29-2	Kite and owl (feces)	*E. coli*	TET, STR, NAL, SXT	Class 1 (*dfrA15-aadA1*)
AZ29-4	Kite and owl (feces)	*M. morganii*	TET, STR, NAL, SXT	Class 1 (*dfrA12-orf-aadA2*)
AZ30-1	Goshawk (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
AZ31-2	Falcon (feces)	*P. vulgaris*	TET, KAN, NAL, AMP, CEF, SXT, CHL	Class 1 (*dfrA1-orf*), $bla_{\text{TEM-1}}$
AZ31-3	Falcon (feces)	*P. mirabilis*	TET, KAN, NAL, AMP, SXT, CHL	Class 1 (*dfrA1-orf*)
AZ33-1	Honey buzzard (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
AZ33-3	Honey buzzard (feces)	*P. mirabilis*	STR, NAL, AMP, SXT, CHL	Class 2 (*dfrA1-sat2-aadA1*) $bla_{\text{TEM-1}}$
AZ35-3	Falcon (feces)	*E. coli*	TET, STR, KAN, CIP, CHL, NOR	Class 1 (*dfrA17-aadA5*)
AZ36-1	Birds (feces)	*E. coli*	AMP, CEF, CPD, CAZ, IMP, NAL, SXT, TET	$bla_{\text{SHV-36}}$
AZ36-4	Wild birds (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
AZ37-1	Wild birds (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
AZ39-1	Japanese four-striped rat snake (feces)	*K. oxytoca*	AMP, CEF, AMC, AZT	$bla_{\text{OXY-2}}$
AZ54-2	Red rat snake (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
AZ58-1	Giant salamander (water)	*K. oxytoca*	AMP, CEF, CPDX, AZT	$bla_{\text{OXY-2}}$
AZ60-2	Eastern box turtle (water)	*K. oxytoca*	NAL	*qnrB*
AZ62-1	Indian star tortoise/feces	*K. pneumoniae*	AMP, NAL, SXT	*qnrB*
AZ66-2	Aldabra giant tortoise (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
AZ67-1	Aldabra giant tortoise (water)	*E. coli*	NAL, TET	*qnrS*
AZ73-2	Colombian rainbow boa (feces)	*K. oxytoca*	AMP, CEF, AMC, AZT	$bla_{\text{OXY-2}}$
AZ74-1	Snowy owl (feces)	*E. coli*	TET, CIP, AMP, CEF, SXT	Class 1 (*dfrA1-aadA1*), *qnrS*, $bla_{\text{TEM-1}}$
AZ75-2	Bengalese finches (feces)	*P. mirabilis*	NAL, TET	*qnrB*
AZ71-1	Elongated tortoise (water)	*E. cloacae*	AMP, CEF, CTX, CRO, CAZ, CPD, IMP, AMC	$bla_{\text{SHV-36}}$
12-6-2	Amur leopard (water)	*C. freundii*	NAL, TET	*qnrB*
21-11-1	Red fox (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$, *qnrS*
25-13-1	Eurasian badger (feces)	*Aeromonas* spp.	AMP, NAL, CIP, NOR, FOX, CHL	*aac(6′)-Ib-cr*
28-14-2	Masked palm civet (feces)	*E. coli*	CPD, CTX, CRO, AMP, CEF, NAL, SXT, STR, CIP, TET	$bla_{\text{CTX-M-2}}$
40-23-1	Reticulated giraffe (feces)	*E. coli*	TET, STR, AMP, SXT	Class 1 (*dfrA1-aadA1*)
49-28-1	Marten (feces)	*E. coli*	STR, SXT	Class 1 (*dfrA2-orf-aadA2*)
52-5-3	Amur tiger (water)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
55-8-3	Wild boar (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
59-11-3	Red fox (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
60-12-3	Racoon dog (feces)	*E. coli*	TET, STR, AMP, SXT	Class 1 (*dfrA5*)
61-13-3	Eurasian badger (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
62-14-3	Masked palm civet (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
73-28-3	Marten (feces)	*E. coli*	AMP, CEF	$bla_{\text{TEM-1}}$
79-13-4	Eurasian badger (feces)	*E. cloacae*	STR, AMP, SXT, NAL	Class 1(*dfrA12-orf-aadA2*) *qnrS*

[a] AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CEF, cephalothin; FOX, cefoxitin; CTT, cefotetan; CFP, cefoperazone; CTX, cefotaxime; CAZ, ceftazidime; CPD, cefpodoxime; CRO, ceftriaxone; ATM, aztreonam; NAL, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; TET, tetracycline; SXT, sulfamethoxazole-trimethoprim.

gene, was identified in two isolates (0.9%), an *E. coli* isolate from birds and an *E. cloacae* isolate from a tortoise (*Indotestudo elongata*) (Table 2). bla_{SHV-36} was detected previously in a clinical isolate of *Klebsiella* spp. isolated from a fecal sample from a hospitalized patient in York, United Kingdom (19), while bla_{SHV-12} was identified previously from *E. coli* isolated from wild birds in Portugal (7). Furthermore, $bla_{CTX-M-2}$, another ESBL-encoding gene, was identified in one *E. coli* isolate from the masked palm civet (Table 2). In Japan, $bla_{CTX-M-2}$ was previously identified in ESBL-producing *E. coli* strains isolated from domestic animals (12, 29). It is worth noting that $bla_{CTX-M-1}$ and $bla_{CTX-M-14}$ have been previously isolated from wild animals in Portugal (7).

Furthermore, this study also identified a novel type of AmpC β-lactamase-encoding gene named bla_{CMY-26}, according to the previously assigned numbers of bla_{CMY}. bla_{CMY-26} was identified in a single isolate of *K. oxytoca* from a jaybird. This *K. oxytoca* strain showed a typical AmpC β-lactamase resistance phenotype, i.e., it was resistant to ampicillin, cephalothin, cefoxitin, cefotetan, ceftriaxone, and amoxicillin-clavulanic acid, in addition to other non-β-lactam antibiotics, such as streptomycin and kanamycin (Table 2). The putative CMY-26 enzyme showed 98% amino acid identity to CMY-13 (accession number AY339625) (18).

In 1998, Martínez-Martínez et al. discovered plasmid-mediated quinolone resistance in a *K. pneumoniae* clinical strain isolate from Alabama (16). The gene responsible for quinolone resistance, *qnr*, encodes a protein of the pentapeptide repeat family, which has been shown to block the action of ciprofloxacin on purified DNA gyrase and topoisomerase IV (30). To date, three main types of *qnr* genes, *qnrA*, *qnrB*, and *qnrS*, have been identified (20, 25). In this study, different primers were used for the screening of the *qnr*-related genes *qnrA*, *qnrB*, and *qnrS*, as described previously (Table 1) (27). A multiplex PCR screening detected *qnr* genes in 10 (4.3%) of the tested isolates and, interestingly, 4 of them were from reptiles (Table 2). DNA sequencing results for the 10 PCR amplicons showed that 6 were *qnrB* and 4 were *qnrS*. The six *qnrB* genes were identified for *E. coli*, *K. pneumoniae*, *K. oxytoca*, *C. freundii*, *P. mirabilis*, and *P. fluorescens* (Table 2). Note that *qnrB* was previously identified for *E. coli* from *K. pneumoniae* in the United States (27) and Korea (21) and from *C. freundii* in Palestine (accession no. AB281054). However, to the best of our knowledge, this is the first report of *qnrB* in *K. oxytoca*, *P. mirabilis*, and *P. fluorescens* and is also the first report of the incidence of *qnrB* in Japan. The four *qnrS* genes were identified from three isolates of *E. coli* and one isolate of *E. cloacae* (Table 2). *qnrS* has been reported previously from human clinical isolates of *E. coli* in France and Scandinavia (4, 24) and has also been detected in clinical isolates of *E. cloacae* from France and Taiwan (24, 32).

More recently, a new mechanism of plasmid-associated quinolone resistance, involving the ciprofloxacin-modifying aminoglycoside acetyltransferase gene, *aac*(*6′*)*-Ib-cr*, has been discovered (26). In this study, universal primers for detection of all types of *aac*(*6′*)*-Ib*, including its variants, were used as described previously (22). PCR and DNA sequencing results identified *aac*(*6′*)*-Ib-cr*, with the typical amino acid substitutions (Trp102Arg and Asp179Tyr) (26), in a single isolate of *Aeromonas* spp. (Table 2). The *aac*(*6′*)*-Ib-cr* gene has been identified previously from *E. coli*, *K. pneumoniae*, and *Enterobacter* sp. isolates in the United States (22). To our knowledge, this is the first report for this gene in Japan.

In summary, the results of the current study highlight zoo animals as a potential reservoir of antimicrobial-resistant bacteria and clinically important resistance genes.

Nucleotide sequence accession number. The nucleotide sequence of the new AmpC β-lactamase gene, bla_{CMY-26}, described in this study was deposited in GenBank under accession no. AB300358.

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